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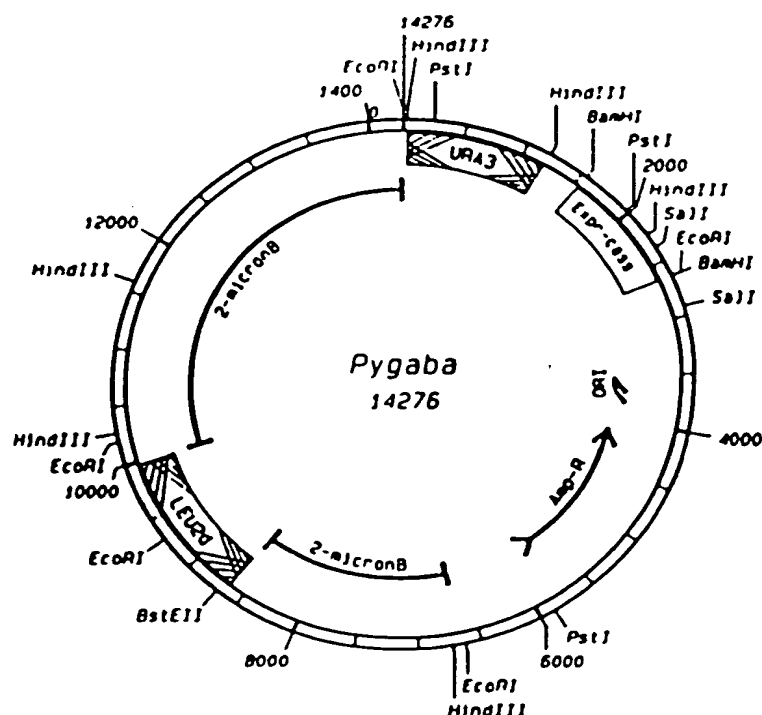
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(54) Title: HUMAN INSULIN ANALOGS AND PREPARATIONS CONTAINING THEM



(57) Abstract

Novel human insulin analogs exhibiting high biological activity and in which the amino acid residue Phe^{B23} is substituted by His or Tyr and moreover substitutions may optionally be present in one or more of the positions A4, A8, A17, A21, B9, B10, B12, B21, B26, B27, B28, and B30, as well as the amino acid residue in the B30 position may further be totally missing or may be blocked at the C-terminal in the form of ester or amide.

Human insulin analogs and preparations containing them.

TECHNICAL FIELD

The present invention relates to novel human insulin analogs exhibiting a high specific biological activity as well as insulin preparations containing the human insulin analogs of the invention.

BACKGROUND ART

Ever since the discovery of insulin in 1922 many different types of insulin preparations have been used for the treatment of Diabetes mellitus. At the beginning exclusively insulin solutions exhibiting a rapidly commencing and relatively rapidly ceasing insulin activity were used, but later on insulin preparations exhibiting a wider profile of activity procured by lowering of the solubility of insulin by means of additions as e.g. zinc salt and/or protamines have been produced. For reasons of availability the insulin used herefor has normally been recovered from Pancreas from domestic animals, most frequently oxes, pigs and sheep, however, recently preparations containing human insulin of biotechnological origin have also appeared on the market.

Throughout the years a large number of artificially prepared analogs of human insulin has been described, usually with the purpose of elucidating the influence of the structure on the activity, vide e.g. Märke et al., Hoppe-Seyler's Z. Physiol. Chem. 360, p. 1619-32 (1979). Investigations of the activity of substitutions of the (B22-B26)-sequence of the insulin on the receptor binding have been of particular interest, as said sequence is considered to be the main field of binding for the insulin receptor, and as naturally occurring mutations have been found with substitutions in said field. Vide e.g. S. Shoelson et al. PNAS 80, p. 7390-94 (1983) and M. Kobayashi et al.: Biomed. Res. 5 (3) p. 267-72 (1984). Very low activities for analogs in which Phe(B24) or Phe(B25)

are substituted are thus found here, and therefore it is concluded that the presence of these two amino acids is of decisive importance to the receptor binding.

Substitutions of the insulin molecule can also be introduced with the purpose of improving the profile of activity of the insulin in the treatment of Diabetes. Thus, e.g. Danish Patent Application No. 5457/86 discloses that one or more substitutions of Glu in the insulin molecule by a neutral amino acid residue causes a shifting of the zone of precipitation of the insulin in such a way that a slow release after injection is obtained.

Moreover, Danish Patent Application No. 4116/86 discloses insulin analogs being particularly rapidly absorbed after injection. This effect is a result of the fact that by means of hydrophilic substitutions in particular in the B9- and in the B28-positions in the insulin molecule a suppression of the aggregation ability of the insulin is obtained so that it is essentially present as monomer. However, a number of these insulin analogs exhibits a reduced biological activity.

20 DISCLOSURES OF THE INVENTION

It has now surprisingly been found that human insulin analogs in which Phe in position B25 is substituted by His or Tyr and optionally further substituted in one or more of the positions A4, A8, A17, A21, B9, B10, B12, B13, B21, B26, B27, B28, and B30, and in which the amino acid residue in position B30 moreover may be missing totally or be blocked at the C-terminal in the form of ester or amide, exhibit a higher biological activity than in such case where Phe in position B25 is not substituted.

30 Accordingly, the present invention relates to human insulin analogs in which the amino acid residue Phe in position B25 is substituted by His or Tyr as well as optionally further substituted in one or more of the positions A4, A8, A17, A21,

B9, B10, B12, B13, B21, B26, B27, B28, and B30, and in which the amino acid residue in position B30 moreover may be missing totally or be blocked at the C-terminal in the form of ester or amide.

5 Examples of particularly preferred substitutions in the above indicated positions are: A4:Gln, A8:His, A17:Gln, A21:Asp, B9:Asp, B10:Asp, B12:Ile, B13:Gln or Arg, B21:Gln or Ile, B26:Glu, B27:Arg, B28:Asp, and B30:Ala or Ser. If several
10 substitutions are desired at the same time in the positions indicated above, it is preferred for reasons of use that they are present within the group: A4, A8, A17, B13, B21, B27, and B30, or within the group: A8, A21, B9, B10, B12, B26, B28, and B30.

Thus, the human insulin analogs of the invention are advantageous in the treatment of Diabetes, as the increased biological activity resulting from the substitution in the B25-
15 -position means a more rapid absorption from the blood and moreover may neutralize the decrease of biological activity which may otherwise be the result of other substitutions in
20 the human insulin molecule.

A particular embodiment of the invention is represented by human insulin analogs containing [His^{B25}] or [Tyr^{B25}] and at least one substitute amino acid residue selected from the group consisting of [Gln^{A4}], [His^{A8}], [Gln^{A17}], [Asp^{A21}],
25 [Asp^{B9}], [Asp^{B10}], [Ile^{B12}], [Gln^{B13}], [Arg^{B13}], [Gln^{B21}], [Pro^{B21}], [Glu^{B26}], [Arg^{B27}], [Asp^{B28}], [Ala^{B30}], and [Ser^{B30}].

Another particular embodiment of the invention is represented by human insulin analogs containing [His^{B25}] or [Tyr^{B25}] and
30 at least two substitute amino acid residues selected from the group consisting of [Gln^{A4}], [His^{A8}], [Gln^{A17}], [Gln^{B13}], [Arg^{B13}], [Gln^{B21}], [Ile^{B21}], [Arg^{B27}], [Ala^{B30}], and [Ser^{B30}].

Yet another particular embodiment of the invention is represented by human insulin analogs containing [His^{B25}] or [Tyr^{B25}] and at least two substitute amino acid residues selected from the group consisting of [His^{A8}], [Asp^{A21}],
5 [Asp^{B9}], [Asp^{B10}], [Ile^{B12}], [Glu^{B26}], [Asp^{B28}], [Ala^{B30}], and [Ser^{B30}].

Preferred human insulin analogs of the invention are
[Tyr^{B25}]-human insulin
[Tyr^{B25}, Asp^{B28}]-human insulin
10 [His^{B25}]-human insulin
[His^{B25}, Asp^{B28}]-des[Thr^{B30}]-human insulin
[Tyr^{B25}]-human insulin-B30-amide
[His^{B25}]-human insulin-B30-amide.

The described insulin derivatives can be produced biosynthetically in yeast expressing a DNA-sequence encoding a given
15 insulin precursor. After the biosynthesis this precursor can be converted into the insulin derivative by an enzymatically catalyzed reaction. To achieve secretion to the growth medium, the DNA-sequence encoding the insulin precursor can be
20 fused to another DNA-sequence encoding a signal peptide functional in yeast. Secretion can be achieved by insertion in the expression plasmid of the *Saccharomyces cerevisiae* MF α 1-leader sequence (Kurjan & Herskowitz, Cell 30, 933-943 (1982)). A preferred construction uses the DNA-sequence en-
25 coding the entire MF α 1-leader sequence including the dibasic site LysArg but excluding the peptide sequence GluAlaGluAla which is the substrate for the yeast protease DPAP (dipeptidyl aminopeptidase). In that way, an efficient secretion of insulin precursors with the correct N-terminal is achieved.

30 DNA-sequences encoding modified insulin precursors were constructed with basis in the expression cassette which is contained in the BamHI restriction fragment from the expression plasmid pYGABA as shown in Figure 1, has a length of 1103 basepairs and contains essentially the following (listed in

succession starting from the 5'-end): The GAPDH promoter (Travis et al., J. Biol. Chem., 260, 4384-4389 (1985) followed by the coding region consisting of: The 83 N-terminal amino acids of the MF α 1-leader sequence encoded by the wild-type yeast DNA sequence as described by Kurjan & Herskowitz (reference given above) followed by the two codons AAA and AGA encoding Lys and Arg and again followed by the coding region for the insulin precursor single chain des[Thr^{B30}]-human insulin (SCI), which is a synthetically constructed gene using preferred yeast codons. After two stop-codons, a SalI restriction site is positioned, and the rest of the sequence constitutes the MF α 1-sequence containing the terminator region. The sequence is constructed using entirely standard techniques.

15 The method employed was "oligonucleotide site directed mutagenesis", which is described by Zoller & Smith, DNA, Vol. 3, No. 6, 479-488 (1984). The method is briefly described in the following, and is described thoroughly in Example 1. Isolated from the expression plasmid the insulin precursor
20 sequence is inserted into a single-stranded, circular M13 bacteriophage vector. To the single-stranded genom, a chemically synthesized complementary DNA-strand is annealed. The DNA-strand contains the desired sequence surrounded by sequences completely homologous to insulin sequences on the
25 circular DNA. In vitro, the primer is then extended in the entire length of the circular genom biochemically using Klenow polymerase. This strand will give rise to single-stranded phages, which when grown in E.coli give the possibility to isolate double-stranded DNA with the desired sequence. From
30 this double-stranded DNA, a restriction fragment can be isolated and reinserted into the expression vector.

Human insulin analogs of the invention in which possible substitutions are only present within the last amino acid residues nearest to the C-terminal of the B-chain may moreover
35 in a manner known per se be prepared semisynthetically from

e.g. porcine insulin as described in K. Inouye et al.:
JACS 101 (3), p. 751-52 (1979), whereby the porcine insulin
is first split with trypsin to des-(B23-30)-human insulin,
whereupon the latter, also enzymatically, is coupled with
5 a synthetic peptide having the desired amino acid sequence.

The invention also relates to insulin preparations which be-
sides the usual adjuvants, excipients and/or carriers com-
prise at least one human insulin analog in which the amino
acid residue in position B25 is His or Tyr, the amino acid
10 residue in one or more of the positions A4, A8, A17, A21,
B9, B10, B12, B13, B21, B26, B27, B28, and B30 is optionally
substituted, and the amino acid residue in the B30-position
is optionally missing or blocked at the C-terminal in the
form of ester or amide. The insulin preparations of the in-
15 vention may be prepared according to conventional methods
for preparing insulin preparations.

MODES FOR CARRYING OUT THE INVENTION

The invention is further illustrated by the following Ex-
amples.

20

EXAMPLE I

Construction of an expression plasmid, which can be used to
express [Tyr^{B25}]-SCI.

The expression cassette, which is contained in the expression
plasmid pYGABA (shown in Figure 1) on a BamHI restriction
25 fragment, was isolated: The expression plasmid was incubated
with the restriction endonuclease BamHI. The conditions were:
20 µg of plasmid, 50 units of BamHI, 100 mM NaCl, 50 mM Tris-
-HCl, pH 7.5, 10 mM MgCl₂, and 1 mM DTT in a volume of 100
µlitres. The temperature was 37°C. and the reaction time 2
30 hours. The two DNA-fragments were separated on a 1% agarose
gel, and the desired fragment was isolated.

Ligation to the M13 vector M13mpl8:

The isolated restriction fragment was ligated to the bacteriophage vector M13mpl8 also cut with the restriction endonuclease BamHI in the following reaction mixture: Fragment 0.2
5 µg, vector 0.02 µg, 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 10 mM DTT and 1 mM ATP in a volume of 20 µlitres. 5 µlitres of this mixture were transformed into the E. coli strain JM101. The presence of fragment in the vector and the orientation of the fragment was determined by restriction enzyme mapping
10 on double-stranded M13-DNA isolated from the transformants.

Transformation of JM101:

The reaction mixture above was transformed in different dilutions into CaCl₂-treated E.coli JM101 cells using standard techniques and plated in 2 x YT topagar on 2 x YT agar plates.
15 (2 x YT = tryptone 16 g/litre, yeast extract 10 g/litre, NaCl 5 g/litre. 2 x YT topagar = 2 x YT with 0.4% agarose added and autoclaved. 2 x YT agar plates = 2 x YT with 2% agar added and autoclaved). The plates were incubated at 37°C. overnight.

20 Identification of positive clones:

The method used was plaque-lift hybridisation which is described in the following: a nitrocellulose-filter was placed on a plate with a suitable plaque-density, so that the filter was wetted. The filter was then bathed in the following
25 solutions: 1.5 M NaCl, 0.5 M NaOH for 30 sec., 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0 for 1 min., 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate) till later use. The filter was dried on 3MM filter paper and baked for 2 hours at 80°C. in a vacuum oven.

30 The mutagenisation primer with the sequence 5'-TGGAGTGTAGTA-GAAACCTCT-3' was labelled radioactively in the 5' end in a 30 µlitres volume containing 70 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 10 pmol oligonucleotide 20 pmol γ-³²P-ATP and 3.5 units of T4 polynucleotide kinase. The mixture was
35 incubated at 37°C. for 30 min. and then for 5 min. at 100°C.

The dried filter was pre-hybridised for 2 hours at 65°C. in 6 x SSC, 0.2% bovine-serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% sodium-dodecyl-sulphate (SDS) and 50 µg/ml salmon-sperm DNA. Then, the reaction mixture containing
5 the labelled probe was added to 15 ml of fresh pre-hybridisation mix, and the filter was bathed herein overnight at 31°C. with gentle shaking. After hybridisation, the filter was washed 3 times for each 15 min. in 2 x SSC + 0.1% SDS and autoradiographed. After wash in the same solution, but
10 now at 61°C., and another autoradiography, plaques containing DNA-sequences complementary to the mutagenisation primer were identified.

Re-screening of positive clones:

Because the identified clone is a result of a heteroduplex,
15 the plaque was plated again. The hybridisation and identification were repeated.

Purification of double-stranded M13-phage DNA:

A re-screened clone was used for infection of the E.coli strain JM101. A culture containing approximately 10^8 phages
20 and 5 colonies of JM101 was grown for 5 hours in a 5 ml 2 x YT medium at 37°C. Then, double-stranded, circular DNA was purified from the pellet according to a method described by Birnboim & Doly, Nucleic Acids Res., 2, 1513 (1979).

Isolation of single-stranded (ss) DNA (template):

25 From the transformant described above ss-DNA was isolated according to a method described by Messing in Gene, 19, 269-276 (1982).

5'-phosphorylation of the mutagenisation primer:

The mutagenisation primer with the sequence 5'-TGGAGTGTAGTA-
30 GAAACCTCT-3' was phosphorylated in the 5'-end in a 30 µl reaction mixture containing 70 mM Tris-HCl, pH 7.0, 10 mM MgCl₂, 5 mM DTT, 1 mM ATP, 100 pmol oligonucleotide and 3.6 units of T4 polynucleotide kinase. The reaction was carried

out for 30 min. at 37°C. Then, the enzyme was inactivated by incubating the mixture for 10 min. at 65°C.

Annealing of template and mutagenisation primer:

Annealing of template and primer was carried out in a 10 µlit-
5 res volume containing 0.5 pmol template, 4 pmol primer, 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM NaCl and 1 mM DTT by heating for 10 min. at 65°C. and cooling afterwards to 0°C.

Extension/ligation reaction:

To the reaction mixture above, 10 µlitres of the following
10 mixture were added: 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, 0.3 mM TTP, 1 mM ATP, 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM DTT, 3 units of T4 DNA ligase and 2.5 units of Klenow polymerase. Then, the reaction was carried out for 16 hours at 16°C.

15 Isolation of a restriction fragment containing modified insulin precursor:

The DNA-preparation (appr. 5 µg) isolated above was digested with 10 units of the restriction endonuclease BamHI in 60
µlitres of 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂,
20 and 1 mM DTT for 2 hours at 37°C. The DNA-products were separated on an agarose-gel, and the fragment was purified from the gel.

Ligation to the yeast vector pAB24 (Figure 2):

The isolated restriction fragment was ligated to the yeast
25 vector pAB24 digested with the restriction endonuclease BamHI in the following reaction mixture: Fragment 0.2 µg, vector 0.02 µg, 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP in a total volume of 20 µlitres. 5 µlitres of this reaction mix was used for transformation of the E.coli strain
30 MC1061, in which the modified expression plasmid was identified and propagated. The plasmid was identical to pYGABA, except for the changed codon.

Transformation of yeast:

Transformation of the expression plasmid into the yeast strain *Saccharomyces cerevisiae* JC482 Δ pep Δ Leu2cir⁰ (α , his4, ura3, leu2, cir⁰) was carried out as described by Ito et al., J. Bact., Vol. 153, No. 1, 163-168 (1983). The transformed cells were plated on SC-ura medium (0.7% Yeast Nitrogen Base, 2.0% glucose, 0.5% casamino acids, 2.0% agar) for selection for plasmid-containing cells.

EXAMPLE II

- 10 Construction of an expression plasmid, which can be used for production of [His^{B25}, Asp^{B28}]-SCI.

The procedure used was essentially the same as described in Example I, except that the mutagenisation primer had the sequence 5'ACAATACCCTTGTCAGTGTAGTGGAACCTCTTT3', that the hybridisation temperature was 42°C., and that the washing temperature after the hybridisation was 63°C. The modified plasmid had a sequence identical to pYGABA, except for the modified codons.

EXAMPLE III

- 20 Expression of precursor and isolation from the culture medium.

Yeast, transformed as described in Example I or II, was propagated on Petri-plates containing minimal-medium without uracil for 48 hours at 30°C. 100 ml shake bottles containing minimal-medium without uracil + 5 g/litre casamino acids + 10 g/litre succinic acid + 30 g/litre glucose at pH 5.0 was inoculated with a single colony from the Petri-plate. The bottles were then shaken at 30°C. in incubator for 72 hours.

After centrifugation 1 litre of pooled supernatant was sterilized by filtration and adjusted to pH 4 - 4.5 and a conductivity < 10 mS by addition of 5 M HCl and water. With a flow

of 120 ml/hour the supernatant was then applied to a 1.6 x 6 cm column of S-Sepharose[®] FF previously equilibrated with 50 mM acetic acid, 50% (by volume) ethanol adjusted to pH 4.0 with NaOH. The column was washed with 60 ml buffer and the precursor was then eluted by a linear gradient of NaCl from 0 to 0.35 M in 360 ml buffer with a flow of 10 ml/hour. The eluate was divided in fractions of 4 ml and detected for UV-absorbance. Fractions containing precursor were identified by RP-HPLC analysis and were pooled. After desalting on a column of Sephadex[®] G25 in 1 M acetic acid the precursor was isolated by lyophilization.

EXAMPLE IV

Preparation of [Tyr^{B25}]-des[Thr^{B30}]-human insulin from precursor.

- 15 450 mg of [Tyr^{B25}]-SCI, prepared by the methods described in Examples I and III, were dissolved in 45 ml of 50 mM tris-(hydroxymethyl)aminomethane, 20% (by volume) ethanol adjusted to pH 7.7 with HCl and 45 ml (settled volume) of Sepharose[®] containing 36 mg of immobilized trypsin in the same buffer were added. The suspension was left for 3 hours at 20°C. with gentle agitation and was then filtered. The gel was washed with 40 ml of buffer, and the pooled filtrates were applied to a 2.6 x 7.5 cm column of Q-Sepharose[®] FF previously equilibrated with 50 mM tris(hydroxymethyl)amino-
25 methane, 50% (by volume) ethanol, adjusted to pH 8.0 with HCl. The column was then eluted with a linear gradient of NaCl from 0 to 0.15 M in the same buffer over 6 hours with a flow of 225 ml/hour. The eluate was detected for UV-absorbance and fractions containing the main protein peak were pooled.
- 30 After dilution with the same volume of water the pool was applied to a 2.6 x 25 cm column of Lichroprep[®] RP-18 (25-40 μ m) previously equilibrated with 10 mM H₃PO₄, 0.1 M NaCl, 30% (by volume) ethanol. With a flow of 16 ml/hour the column

was then eluted with a linear gradient of ethanol from 30% to 40% (by volume) over 20 hours. The eluate was detected for UV-absorbance and fractions containing the main protein peak were pooled. After desalting on a column of Sephadex[®] G25 in 1 M acetic acid 105 mg of [Tyr^{B25}]-des[Thr^{B30}]-human insulin were isolated by lyophilization.

The protein was redissolved in a mixture containing 200 mg of threonine methyl ester, 1.0 ml of ethanol and 0.4 ml of water. The pH value was adjusted to 6.3 with acetic acid, and 2 ml (settled volume) of Sepharose[®] containing 1.6 mg of immobilized trypsin were added. After standing for 2 hours at 20°C. with gentle agitation, the gel was removed by filtration, and the protein was precipitated by addition of 10 volumes of 2-propanol. The air-dried precipitate was redissolved in 20 mM tris(hydroxymethyl)aminomethane/HCl, 60% (by volume) ethanol, pH 8.25, and applied to a 1.6 x 20 cm Q-Sepharose[®] FF column, previously equilibrated with the said buffer, and eluted with a linear NaCl-gradient in the same buffer increasing from 0 to 0.1 M over 15 hours at a flow rate of 50 ml/hour. The ethanol was removed in vacuo from the pooled fractions containing [Tyr^{B25}]human insulin-(B30-methyl ester), and the protein was precipitated by adjusting the pH value to 6.1. After centrifugation and lyophilization the methyl ester was hydrolyzed for 10 min. in cold 0.1 M NaOH at a protein concentration of 10 mg/ml. The reaction was stopped by adjusting the pH value to 8.5, and 2 volumes of 20 mM tris(hydroxymethyl)aminomethane/HCl, pH 8.5, were added. The solution was then applied to a 1.6 x 20 cm Q-Sepharose[®] FF column and eluted as described above. The protein was precipitated at a pH value of 5.5 after removal of the ethanol in vacuo. 40 mg of [Tyr^{B25}]-human insulin were obtained after lyophilization.

The identity of the product was confirmed by amino acid analysis and by sequential Edman degradation of the separated vinylpyridylated A- and B-chains.

EXAMPLE V

Preparation of $[\text{His}^{\text{B25}}, \text{Asp}^{\text{B28}}]\text{-des}[\text{Thr}^{\text{B30}}]\text{-human insulin}$ from precursor.

- 100 mg of $[\text{His}^{\text{B25}}, \text{Asp}^{\text{B28}}]\text{-SCI}$, prepared by the methods described in Examples II and III, were dissolved in 10 ml of 50 mM tris(hydroxymethyl)aminomethane, 20% (by volume) ethanol, adjusted to pH 7.7 with HCl and 10 ml (settled volume) of Sepharose [®], containing 8 mg of immobilized trypsin, in the same buffer were added. The suspension was left for 3 hours at 20°C. with gentle agitation and was then filtered. The gel was washed with 10 ml of buffer, and the pooled filtrates were applied to a 1.6 x 7.5 cm column of Q-Sepharose [®] FF previously equilibrated with 50 mM tris(hydroxymethyl)-aminomethane, 50% (by volume) ethanol, adjusted to pH 8.0 with HCl. The column was then eluted with a linear gradient of NaCl from 0 to 0.15 M in the same buffer over 6 hours with a flow of 90 ml/hour. The eluate was detected for UV-absorbance, and fractions containing the main protein peak were pooled.
- After dilution with the same volume of water the pool was applied to a 1.6 x 25 cm column of Lichroprep [®] RP-18 (25-40 μm) previously equilibrated with 10 mM H_3PO_4 , 0.1 M NaCl, 30% (by volume) ethanol. With a flow of 6.5 ml/hour the column was then eluted with a linear gradient of ethanol from 30% to 40% (by volume) over 20 hours. The eluate was detected for UV-absorbance, and fractions containing the main protein peak were pooled. After desalting on a column of Sephadex [®] G25 in 1 M acetic acid 39 mg of $[\text{His}^{\text{B25}}, \text{Asp}^{\text{B28}}]\text{-des}[\text{Thr}^{\text{B30}}]\text{-human insulin}$ were isolated by lyophilization.
- The identity of the product was confirmed by amino acid analysis and by sequential Edman degradation of the separated vinylpyridylated A- and B-chains.

EXAMPLE VI

Preparation of des-(B23-B30)-human insulin.

1 g of Na-crystallized porcine insulin (protein weight) was dissolved in 40 ml of water, and a solution of 50 mg of porcine trypsin in 10 ml of freshly prepared 0.25 M ammonium hydrogencarbonate solution in which pH has been adjusted to 9.0 with ammonia water was added. Then, the solution was left in a refrigerator at 4°C. After 48 hours a HPLC analysis showed a degree of reaction of 65%. Thereafter the solution was gel-filtered at 4°C. on a 5 x 90 cm column of Sephadex G50 Superfine in 0.05 M ammonium hydrogencarbonate at a flow of 90 ml/hour. Fractions containing the main protein peak were pooled and lyophilized.

Yield: 520 mg of des-(B23-30)-human insulin of a purity of 97.5%, measured by HPLC analysis.

EXAMPLE VII

Preparation of peptides.

Peptides were prepared by means of a peptide synthesis machine from Applied Biosystems, whereby the construction was made on a PAM resin by means of protected symmetrical amino acid anhydrides. The capacity was about 0.1 mmole of peptide. Finally, the peptide was split from the resin by reaction with anhydrous hydrogen fluoride at 0°C., whereby the remaining protection groups were simultaneously removed.

EXAMPLE VIII

Preparation of [His^{B25}]-human insulin.

200 mg of des-(B23-30)-human insulin and 400 mg of Gly-Phe-His-Tyr-Thr-Pro-Lys-Thr were dissolved in a mixture

of 2.40 ml of dimethyl formamide and 1.20 ml of water, the pH of the mixture being adjusted to 6.5 with triethyl amine.

Then, a solution of 10 mg of porcine trypsin in 0.20 ml of water was added, and the reaction mixture was left at 20°C. After 4 hours a HPLC analysis showed a reaction of 60%, and the reaction was stopped by precipitation with 25 ml of 2-propanol. The precipitate which was isolated by centrifugation was redissolved in 10 ml of 1 M acetic acid and applied at 20°C. to a 2.6 x 20 cm column of Lichroprep[®] RP-18 (25-40 µm), equilibrated in a buffer consisting of 0.5 mM hydrogenchloride, 0.1 M sodium chloride in 30% (by volume) ethanol. Then the column was eluted with the same buffer at a flow of 20 ml/hour, the content of ethanol however being linearly increased to 50% over 24 hours. The fractions containing the main protein peak were pooled, whereupon the protein was precipitated by dilution with the same volume of water and adjustment of pH to 5.5 with sodium hydroxide solution. After standing at 4°C. for 1 hour the suspension was centrifuged, and the precipitate was freeze-dried. Hereby 90 mg of [His^{B25}]-human insulin were obtained, identified by total amino acid analysis and by sequential Edman degradation of the separated vinylpyridylated A- and B-chains.

EXAMPLE IX

Preparation of [Tyr^{B25}, Asp^{B28}]-human insulin.

150 mg of des-(B23-30)-human insulin and 350 mg of Gly-Phe-Tyr-Tyr-Thr-Asp-Lys-Thr were dissolved in a mixture of 2.0 ml of dimethyl formamide and 1.0 ml of water, the pH being adjusted to 6.5 with triethyl amine. Now a solution of 8 mg of porcine trypsin in 0.20 ml of 1 mM calcium acetate solution was added, and the reaction mixture was left at 15°C. After 3 hours a HPLC analysis showed a reaction of 50%, and the reaction was stopped by adding 25 ml of 2-propanol.

After centrifugation the precipitate was redissolved in 10 ml of 1 M acetic acid and applied at 20°C. to a 2.6 x 20 cm column of Lichroprep[®] RP-18 (25-40 µm), equilibrated in a buffer consisting of 0.5 mM hydrogenchloride, 0.1 M sodium chloride in 30% (by volume) ethanol. Then, the column was eluted with the same buffer at a flow of 20 ml/hour, the content of ethanol however being linearly increased to 50% over 24 hours. The fractions containing the main protein peak were pooled, whereupon the protein was precipitated by dilution with the same volume of water and adjustment of pH to 5.0 with sodium hydroxide solution. After standing overnight at 4°C. the precipitate was isolated by centrifugation and freeze-dried.

Yield: 60 mg of [Tyr^{B25}, Asp^{B28}]-human insulin, identified by total amino acid analysis and by sequential Edman degradation of the separated vinylpyridylated A- and B-chains.

EXAMPLE X

Evaluation of biological activity.

The biological activity in vitro was determined by measuring the binding affinity to the insulin receptors of isolated rat adipocytes and hepatocytes essentially as described in J. Gliemann, S. Gammeltoft: Diabetologia 10, 105-113 (1974).

The insulin analogs were compared to semisynthetic human insulin, the potency of which was set to 100%, and the results are shown in the Table below:

	Adipocytes	Hepatocytes
[Tyr ^{B25}]-human insulin	356%	222%
[Tyr ^{B25} , Asp ^{B28}]-human insulin	201%	138%
[His ^{B25}]-human insulin	150%	142%
30 [His ^{B25} , Asp ^{B28}]-des[Thr ^{B30}]-human insulin	130%	135%

CLAIMS

1. Human insulin analogs exhibiting high biological activity, characterized in that the amino acid residue in position B25 is His or Tyr, that the amino acid residue in one or more of the positions A4, A8, A17, A21, B9, B10, B12, B13, B21, B26, B27, B28, and B30 is optionally substituted, and that the amino acid residue in the B30-position is optionally missing or blocked at the C-terminal in the form of ester or amide.
2. Human insulin analogs according to claim 1, characterized by containing [His^{B25}] or [Tyr^{B25}] and at least one substitute amino acid residue selected from the group consisting of [Gln^{A4}], [His^{A8}], [Gln^{A17}], [Asp^{A21}], [Asp^{B9}], [Asp^{B10}], [Ile^{B12}], [Gln^{B13}], [Arg^{B13}], [Gln^{B21}], [Pro^{B21}], [Glu^{B26}], [Arg^{B27}], [Asp^{B28}], [Ala^{B30}], and [Ser^{B30}].
3. Human insulin analogs according to claim 1, characterized by containing [His^{B25}] or [Tyr^{B25}] and at least two substitute amino acid residues selected from the group consisting of [Gln^{A4}], [His^{A8}], [Gln^{A17}], [Gln^{B13}], [Arg^{B13}], [Gln^{B21}], [Ile^{B21}], [Arg^{B27}], [Ala^{B30}], and [Ser^{B30}].
4. Human insulin analogs according to claim 1, characterized by containing [His^{B25}] or [Tyr^{B25}] and at least two substitute amino acid residues selected from the group consisting of [His^{A8}], [Asp^{A21}], [Asp^{B9}], [Asp^{B10}], [Ile^{B12}], [Glu^{B26}], [Asp^{B28}], [Ala^{B30}], and [Ser^{B30}].
5. Human insulin analog according to claim 1, characterized by being [Tyr^{B25}]-human insulin.
6. Human insulin analog according to claim 1, characterized by being [Tyr^{B25}, Asp^{B28}]-human insulin.
7. Human insulin analog according to claim 1, characterized

ter i z e d by being [His^{B25}]-human insulin.

8. Human insulin analog according to claim 1, c h a r a c -
t e r i z e d by being [His^{B25}, Asp^{B28}]-des[Thr^{B30}]-human
insulin.

5 9. Human insulin analog according to claim 1, c h a r a c -
t e r i z e d by being [Tyr^{B25}]-human insulin-B30-amide.

10. Human insulin analog according to claim 1, c h a r a c -
t e r i z e d by being [His^{B25}]-human insulin-B30-amide.

11. Insulin preparation, c h a r a c t e r i z e d by con-
10 taining at least one human insulin analog in which the amino
acid residue in position B25 is His or Tyr, the amino acid
residue in one or more of the positions A4, A8, A17, A21, B9,
B10, B12, B13, B21, B26, B27, B28, and B30 is optionally sub-
stituted, and the amino acid residue in the B30-position is
15 optionally missing or blocked at the C-terminal in the form
of ester or amide.

12. Insulin preparation according to claim 11, c h a r a c -
t e r i z e d by containing at least one human insulin ana-
log comprising [His^{B25}] or [Tyr^{B25}] and at least one substi-
20 tute amino acid residue selected from the group consisting
of [Gln^{A4}], [His^{A8}], [Gln^{A17}], [Asp^{A21}], [Asp^{B9}], [Asp^{B10}],
[Ile^{B12}], [Gln^{B13}], [Arg^{B13}], [Gln^{B21}], [Ile^{B21}], [Glu^{B26}],
[Arg^{B27}], [Asp^{B28}], [Ala^{B30}], and [Ser^{B30}].

13. Insulin preparation according to claim 11, c h a r a c -
25 t e r i z e d by containing at least one human insulin ana-
log comprising [His^{B25}] or [Tyr^{B25}] and at least two substi-
tute amino acid residues selected from the group consisting
of [Gln^{A4}], [His^{A8}], [Gln^{A17}], [Gln^{B13}], [Arg^{B13}], [Gln^{B21}],
[Ile^{B21}], [Arg^{B27}], [Ala^{B30}], and [Ser^{B30}].

30 14. Insulin preparation according to claim 11, c h a r a c -

t e r i z e d by containing at least one human insulin analog comprising [His^{B25}] or [Tyr^{B25}] and at least two substitute amino acid residues selected from the group consisting of [His^{A8}], [Asp^{A21}], [Asp^{B9}], [Asp^{B10}], [Ile^{B12}], [Glu^{B26}],
5 [Asp^{B28}], [Ala^{B30}], and [Ser^{B30}].

15. Insulin preparation according to claim 11, c h a r a c -
t e r i z e d by containing [Tyr^{B25}]-human insulin.

16. Insulin preparation according to claim 11, c h a r a c -
t e r i z e d by containing [Tyr^{B25}, Asp^{B28}]-human insulin.

10 17. Insulin preparation according to claim 11, c h a r a c -
t e r i z e d by containing [His^{B25}]-human insulin.

18. Insulin preparation according to claim 11, c h a r a c -
t e r i z e d by containing [His^{B25}, Asp^{B28}]-des[Thr^{B30}]-
-human insulin.

15 19. Insulin preparation according to claim 11, c h a r a c -
t e r i z e d by containing [Tyr^{B25}]-human insulin-B30-
-amide.

20. Insulin preparation according to claim 11, c h a r a c -
t e r i z e d by containing [His^{B25}]-human insulin-B30-amide.

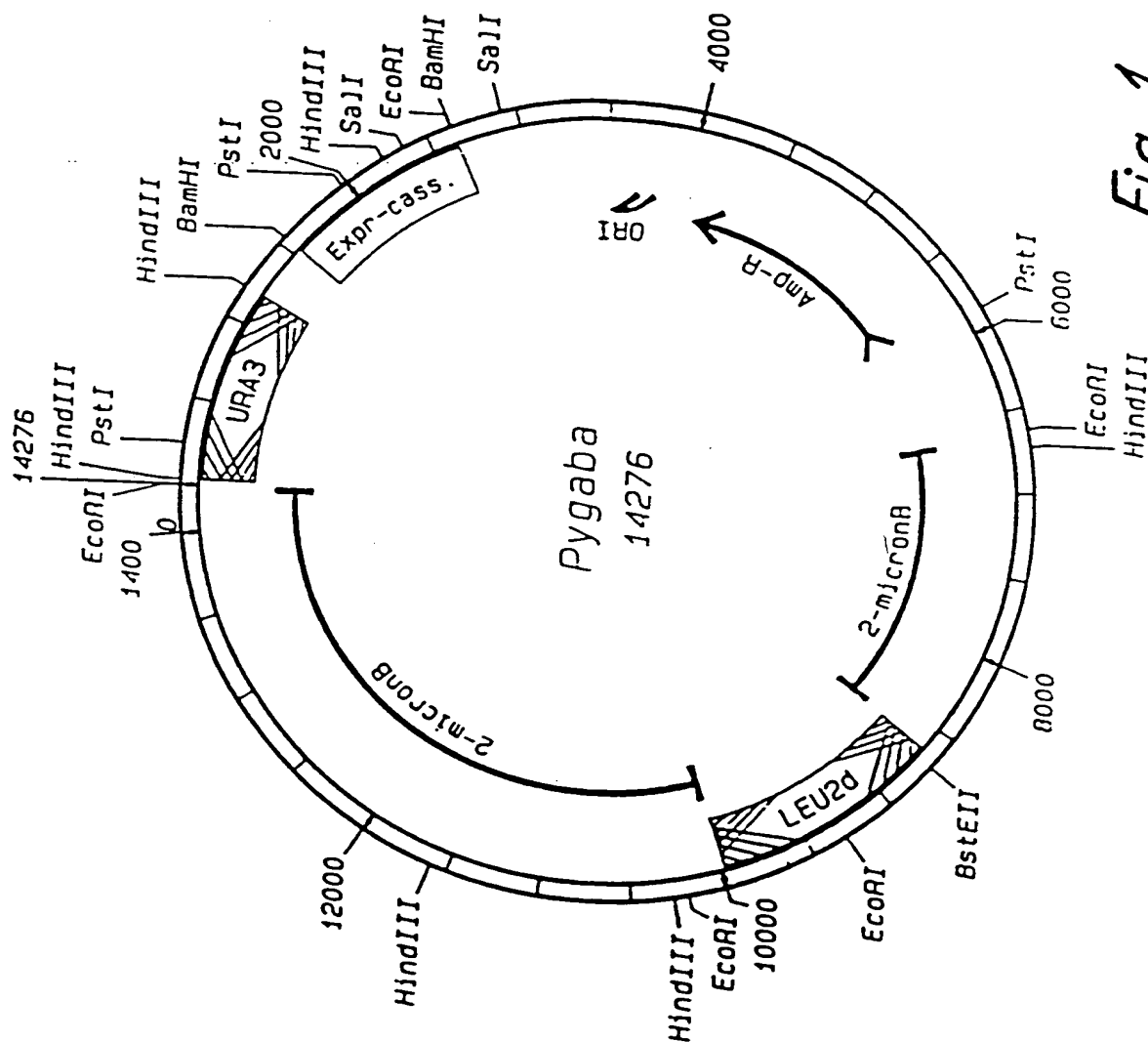


Fig. 1

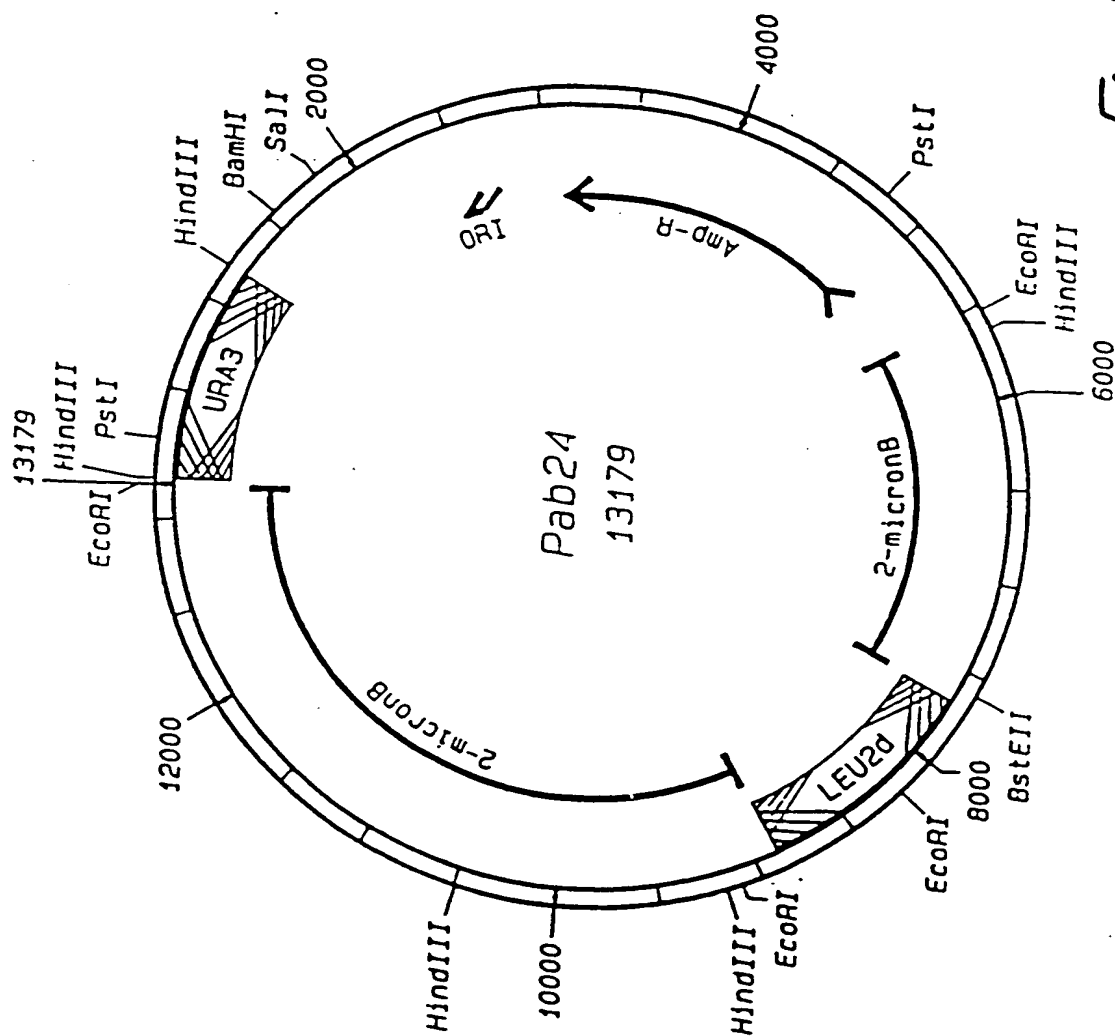
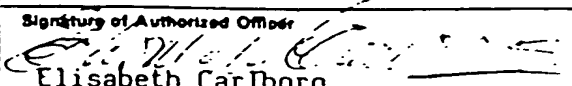


Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No. PCT/DK88/00124

I. CLASSIFICATION & SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC 4		
C 07 K 7/40; A 61 K 37/26 //C 12 N 15/00; C 12 P 21/02; C 07 K 99:26		
II. FIELDS SEARCHED		
Minimum Documentation Searched 7		
Classification System	Classification Symbols	
IPC	A 61 K 37/26; C 07 K 7/40; C 12 N 15/00; C 12 P 21/02	
US C1	260:112.7; 424:178; 514:3; 530:303	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8		
SE, NO, DK, FI classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT 9		
Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	EP, A2, O 194 864 (NOVO INDUSTRI A/S) 17 September 1986 & JP, 61212598	1-20
Y	EP, A2, O 214 826 (NOVO INDUSTRI A/S) 18 March 1987 & JP, 62053999	1-20
Y	EP, A2, O 254 516 (NOVO INDUSTRI A/S) 27 January 1988 & JP, 63099096 ZA, 8705295	1-20
Y	WO, A1, 86/05496 (NORDISK GENTOFTE A/S) 25 September 1986 & WO, 86/05497 EP, 216832 EP, 216833 JPT, 62502196 JPT, 62502538	1-20
.../...		
<p>* Special categories of cited documents: 10</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
1989-03-01	1989-03-06	
International Searching Authority	Signature of Authorized Officer	
Swedish Patent Office	 Elisabeth Carlborg	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	WO, A1, 86/05497 (NORDISK GENTOFTE A/S) 25 September 1986 & WO, 86/05496 EP, 216832 EP, 216833 JPT, 62502196 JPT, 62502538	1-20
Y	WO, A1, 88/02005 (NORDISK GENTOFTE A/S) 24 March 1988 & EP, 282550	1-20
X	Peptides 1980, Proceedings of the Sixteenth European Peptide Symposium Helsingør, Denmark, August 31-September 6, 1980, Copenhagen 1981, Ed. K BRUNFELDT; Trypsin Catalyzed Peptide Synthesis: Modification of the B-chain C-terminal region of insulin, GATTNER H-G et al, p. 372-377	1-6 9-10
Y	Hoppe-Seyler's Z. Physiol. Chem. Bd 360, s. 1619-1632, November 1979, Fritz Mäki et al, "Synthesis and Biological Activity of Seventeen Analogues of Human Insulin".	1-4
Y	Chemical Abstracts, Vol. 107 (1987), abstract No. 127328W, Biol. Chem. Hoppe-Seyler 1987, 368(6), 709-716 (Eng).	1-20